Homogenization of insect bulk samples yields more comprehensive yet comparable biodiversity data than non-destructive lysis

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Abstract

The rapid advancement of molecular biodiversity monitoring tools, particularly DNA metabarcoding, has improved specimen identification in bulk samples, such as those from Malaise traps, where traditional morphological identification is impractical. While not yet standardized, a typical first step in insect bulk sample analysis is the extraction of DNA from homogenized specimens. While this step yields reliable metabarcoding results, it destroys the specimens, preventing further use in monitoring and taxonomic analysis. Non-destructive lysis, which preserves specimen integrity, is still being evaluated for its effectiveness in accurately assessing bulk sample biodiversity. In this study, we assessed the suitability of non-destructive lysis for Malaise trap samples and compared its performance with homogenization using an established metabarcoding workflow. Five bulk samples were collected with Malaise traps. Samples were first incubated in a lysis buffer containing Proteinase K (non-destructive lysis) and then homogenized. DNA was extracted from both treatments and metabarcoding was performed to compare OTU richness, accumulation, and beta diversity. On average, homogenized samples yielded 3.8% more OTUs than non-destructive lysis samples. Although homogenization provides a more comprehensive and cost-effective assessment of Malaise trap bulk samples, non-destructive lysis still recovered at least 80% of the OTUs identified through homogenization and revealed similar patterns of community change. Even though our results show that both methods yield comparable data on insect biodiversity and can be used for monitoring, we consider non-destructive lysis as not suitable for integration into automated workflows or large-scale biomonitoring due to the much higher costs. Nonetheless, this method remains important in cases where morphological integrity needs to be preserved and additional sampling is not possible.

Key words: Biodiversity monitoring, bulk sample, homogenization, insects, Malaise trap, metabarcoding, non-destructive lysis

Introduction

In the last two decades, tools for molecular biodiversity monitoring have rapidly evolved. In particular, DNA metabarcoding has facilitated and accelerated the identification of specimens in bulk samples, also known as "biodiversity soup"

(Yu et al. 2012). This acceleration is essential since samples often contain thousands of specimens and it is therefore impossible to identify specimens within the time frame required for bioassessments and biodiversity monitoring. This limitation of morphological identification becomes prevalent, especially for specimen-rich insect samples, e.g. those obtained with Malaise traps that can collect over ten thousand specimens within a single week (Geiger et al. 2016; Karlsson et al. 2020; Lehmann et al. 2021; Remmel et al. 2024). Thus, DNA metabarcoding is increasingly being used as a tool to assess species diversity in Malaise trap samples (Steinke et al. 2022; Zizka et al. 2022).

A reliable source material for metabarcoding is DNA extracted from completely homogenized insect bulk samples (Hausmann et al. 2020; Buchner et al. 2023). However, the destruction of specimens makes it impossible to retain vouchers and is therefore not applicable in cases where specimens need to be retained, e.g. for quality control in regulatory monitoring, subsequent phenotypic analysis, or species descriptions. Therefore, two different metabarcoding approaches retaining the morphological integrity of specimens have been proposed: i) analysis of the sample community via the storage ethanol (Erdozain et al. 2019; Kirse et al. 2022) and ii) non-destructive lysis of bulk samples, during which samples are incubated in a lysis buffer to obtain insect DNA without homogenization (Batovska et al. 2021; Marquina et al. 2022; Iwaszkiewicz-Eggebrecht et al. 2023). The analysis of preservative ethanol has not proven to be a reliable tool for biodiversity assessment in terrestrial arthropod bulk samples (Marquina et al. 2019; Chimeno et al. 2023), whereas non-destructive lysis of insect samples has shown potential to adequately reflect their biodiversity (Carew et al. 2018; Kirse et al. 2022).

Many of these validation studies are based on artificially composed mock communities (Batovska et al. 2021; Marquina et al. 2022; Martoni 2022), which contain fewer species and specimens than typical Malaise trap samples, so that the results of these methods cannot be compared without further testing. In a study that used the large diversity of Malaise trap samples (Kirse et al. 2022) the treatments of homogenized samples and those treated with non-destructive lysis were not identical, as they differed in lysate volumes and extraction methods, which thus limits direct comparisons. However, for method comparison and validation, the treatments should ideally be equal for both methods varying only test parameters - in this case this means that the same sample should first be treated with non-destructive lysis and subsequently homogenized. The two sample types should then be processed with identical volumes and laboratory protocols. Consequently, there is a clear need to directly compare the performance of bulk sample metabarcoding with specimen-rich samples using non-destructive lysis and homogenized bulk material to develop method standardization recommendations for insect biodiversity monitoring.

Therefore, this study aimed to (i) evaluate the suitability of a non-destructive lysis approach applied to Malaise trap samples as well as to (ii) compare the performance of non-destructive lysis and homogenization for the same sample with identical metabarcoding workflows that are already well established and scalable.

We hypothesize that homogenized bulk samples yield higher diversity since DNA will be more accessible after homogenization compared to non-destructive lysis.

Material and methods

Sample collection

Five insect bulk samples were collected using Malaise traps of the LTER type (Soltwedel 2022) from May to June 2022 near the River Boye in Bottrop, Germany. Samples were collected weekly, with insects initially caught in 70% ethanol and subsequently preserved in 96% ethanol. For use in a separate study, all specimens of the family Syrphidae (Diptera) and the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) were removed from the samples prior to analysis.

Laboratory processing

For the laboratory workflow (Fig. 1), the fixative ethanol was removed from the samples by draining over a sieve (250 µM mesh size) until no more drops leaked from the inverted sample. The samples were then weighed and subsequently, TNES buffer (Buchner 2022d) was added to the samples until all insects were submerged (140–180 mL, Suppl. material 2). For each mL of TNES buffer, 100 µL of Proteinase K stock solution (10 mg/mL) was added to each sample.

The samples were then incubated at 56 °C for 18 hours. Afterwards, the lysis buffer was drained from the samples with a 250 µM sieve and stored for subsequent analyses. The remaining bulk samples were filled up with 96% ethanol. After short mixing through inversion, the ethanol was immediately exchanged with fresh 96% ethanol to ensure the complete removal of TNES buffer. To ensure equal conditions among the treatments, an identical volume of ethanol was used for each sample as the volume of TNES buffer previously used in the non-destructive lysis. The samples were then homogenized wet with a tissue tearer (BioSpec Products, Bartlesville, USA) for 2 minutes. The tissue tearer was cleaned in 3% bleach in between samples under a fume hood (Buchner et al. 2021a).

From the TNES buffer removed from each sample, 25 subsamples with a volume of 1 mL each were transferred into 2 mL microcentrifuge tubes. All subsequent steps were performed on an automated liquid handling robot (Hamilton Vantage, Hamilton Bonaduz AG, Switzerland). Identical to the non-destructive lysis procedure, 25 subsamples of 1 mL homogenate were taken from each of the five bulk samples. The tissue was pelleted via centrifugation at 4.000× g for 3 minutes. The remaining ethanol was removed and lysis was conducted through bead-beating in 1 mL lysis buffer with the same concentration as in the non-destructive lysis protocol (Buchner 2022d). The lysis of homogenate was performed on a shaking incubator at 56 °C for 20 min at 1400 rpm. The samples were subsequently handled on an automated liquid handling robot (Hamilton Vantage, Hamilton Bonaduz AG, Switzerland). For all samples, regardless of treatment, remaining debris in the samples was pelleted via centrifugation at 4.000× g for 3 minutes. The samples were then transferred into 96-well plates in 2 technical replicates with 300 µL of lysate each according to the workflow proposed in Buchner et al. 2021b with 12 negative controls per plate.

DNA was extracted with a silica spin column-based protocol (Buchner 2022a) via a vacuum manifold. Extraction success was checked on a 1% agarose gel. Samples were amplified in a first PCR with the Qiagen Multiplex Plus Kit (Qiagen, Hilden, Germany) with a final concentration of 1× Multiplex Mastermix, 200 nM of each primer (fwh2F, fwhR2n, Vamos et al. (2017)) filled up to a total volume
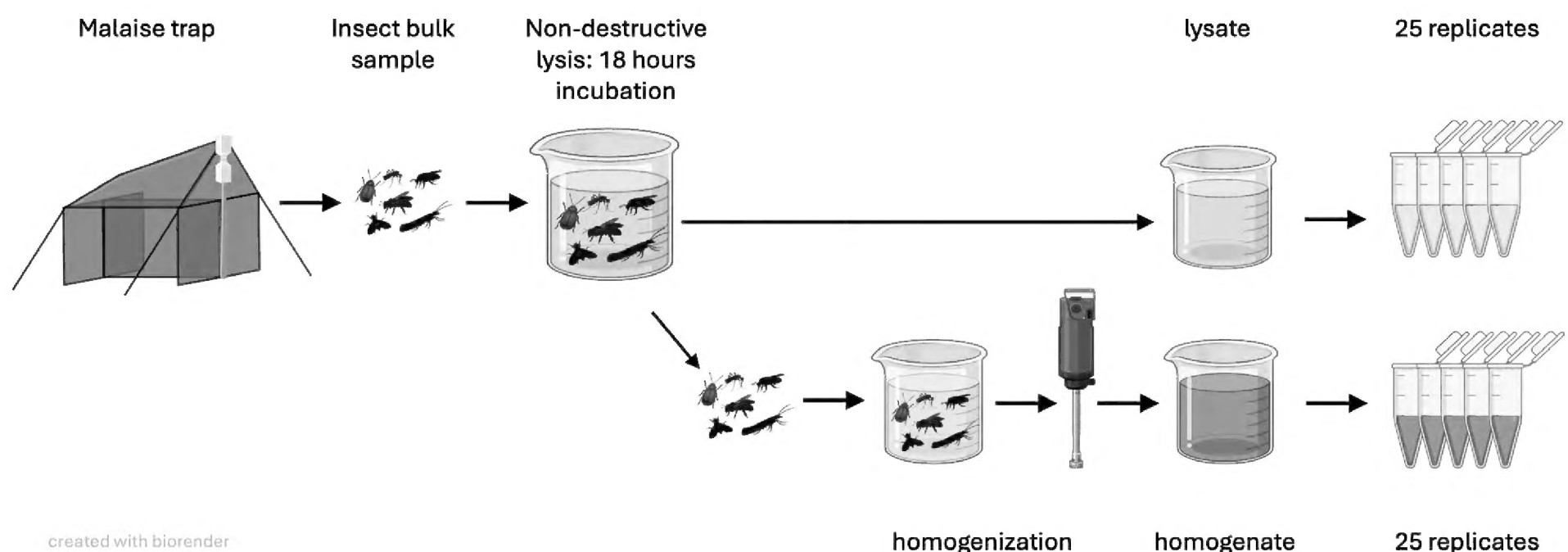
Figure 1. Laboratory processing of insect bulk samples. Samples were incubated in lysis buffer for 18 h. From the lysis buffer 25 replicates were taken for non-destructive lysis processing (top, blue). In parallel, the incubated specimens were homogenized and 25 replicates were taken from the homogenate (bottom row, red).

of 10 µL with PCR-grade water. Cycling conditions were as follows: Initial denaturation for 5 min at 95 °C, 20 cycles of denaturation (95 °C, 0:30 min) annealing (58 °C, 1:30 min) and extension (72 °C, 0:30 min) finished with a final elongation step at 68 °C for 10:00 min. Each of the PCR plates used in the first PCR was tagged with a unique combination of inline tags. Additionally, the primer contained a universal binding site for the primer used in the second PCR step to anneal (Suppl. material 3). A bead-based cleanup was conducted in between the two PCR steps (Buchner 2022b). For the second, PCR samples were amplified with a final concentration of 1× Multiplex Mastermix, 1× CoralLoad loading dye and 100 nM of each primer. Each of the wells was individually tagged with Illumina indices with the following cycling conditions: Initial denaturation for 5:00 min at 95 °C, 25 cycles of denaturation (95 °C, 0:30 min) annealing (61 °C, 1:30 min) and extension (72 °C, 0:30 min) and a final elongation at 68 °C for 10:00 min. PCR success was checked on a 1% agarose gel. PCR products were normalized with a bead-based protocol (Buchner 2022c). To remove overamplified products and remaining primer dimers, a reconditioning PCR was performed (Initial denaturation at 95 °C for 05:00 min, one cycle of denaturation at 95 °C for 30 sec, annealing at 60 °C for 1:30 min, and extension at 72 °C for 1:30 min and a final elongation at 68 °C for 10:00 min) (Buchner 2024). Finally, library quality was checked with a Fragment analyzer (High sensitivity NGS Fragment Analysis Kit; Advanced analytical, Ankeny, USA).

Sequencing was performed on an Illumina NovaSeq 6000 instrument with 300 cycles (paired-end 2 × 150 bp) at GENEWIZ, Leipzig, Germany.

Bioinformatic analysis

Raw data of the sequencing runs were delivered demultiplexed by index read. Additional demultiplexing of the inline tags was achieved with the Python package "demultiplexer" (v1.2.0). Sequencing data were then processed with the APSCALE pipeline (Buchner et al. 2022), including paired-end merging, primer trimming, quality filtering, dereplication, OTU clustering and lulu filtering according to default settings. Sequences were clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold. The subsequent taxonomic

assignment was performed with the Barcode of Life database (Ratnasingham and Hebert 2007) using BOLDigger v. 2 (Buchner and Leese 2020). The analyses of richness and similarity were based on the identified OTUs, irrespective of their species matches in the Barcode of life Database. As an additional filtering step, only reads detected in both technical replicates were accepted as true signals and these reads were subsequently summed, leading to a final number of 25 replicates per sample used in the subsequent analysis. The reads in all replicates were rarefied to the number of reads in the replicate with the lowest read count per sample through randomized subsampling, ensuring equal sequencing depth for all replicates in the final OTU table (Suppl. material 4). The effect of the treatment (non-destructive lysis or homogenization) on OTU richness was estimated with a linear model in Python with the statsmodels module. Richness per treatment box-plots were created in Python seaborn and richness differences per sample were estimated using a Mann-Whitney-U test. An OTU accumulation curve was created with a custom Python script (Suppl. material 1) to simulate subsequent addition of replicates and the resulting increase in OTU richness per added replicate. For this purpose, the respective combination of replicates was drawn from the pool of all possible combinations 1000 times at maximum and all possible combinations were drawn in low replicate numbers in which the maximum of possible combinations was below 1000. For a principal component analysis (PCA), Jaccard beta diversity distances were calculated as a distance measure between samples. The full Python code for this analysis is provided in Suppl. material 1.

Results

After the 18-hour non-destructive lysis treatment in TNES and Proteinase K, the specimens were already very soft and their suitability for individual handling for morphological determination was extremely limited.

After subsampling according to the minimum reads per sample, the resulting sequencing depths were as follows: sample 1 with 406,080 reads, sample 2 with 391,897 reads, sample 3 with 519,754 reads, sample 4 with 502,793 reads, and sample 5 with 331,831 reads.

The non-destructive lysis treatment recovered significantly fewer OTUs in four out of five samples (Mann-Whitney-U test, $p < 0.001$ in all samples, Fig. 2a–e). In comparison, 3.8% more OTUs were recovered through homogenization than by non-destructive lysis of the same samples.

The analysis of the OTU accumulation curve with increased replication showed that in three out of five samples the number of detected OTUs was higher in homogenate samples even with a low number of replicates. Furthermore, additional replicates did not increase the richness recovered from non-destructive lysis samples to a level comparable to that of the homogenate samples.

The ordination analysis based on Jaccard diversity showed that both methods recovered systemically different communities separated through axis 1 and that variation between replicates among technical replicates (axis 2) was very low (Fig. 2k–o). Additionally, in three samples, the homogenate showed higher within-treatment similarity than the non-destructive lysis samples, i.e. a higher variability in communities recovered from identical samples by non-destructive lysis.

The number of OTUs exclusively detected in homogenized samples was on average 75% higher compared to those detected in non-destructive lysis
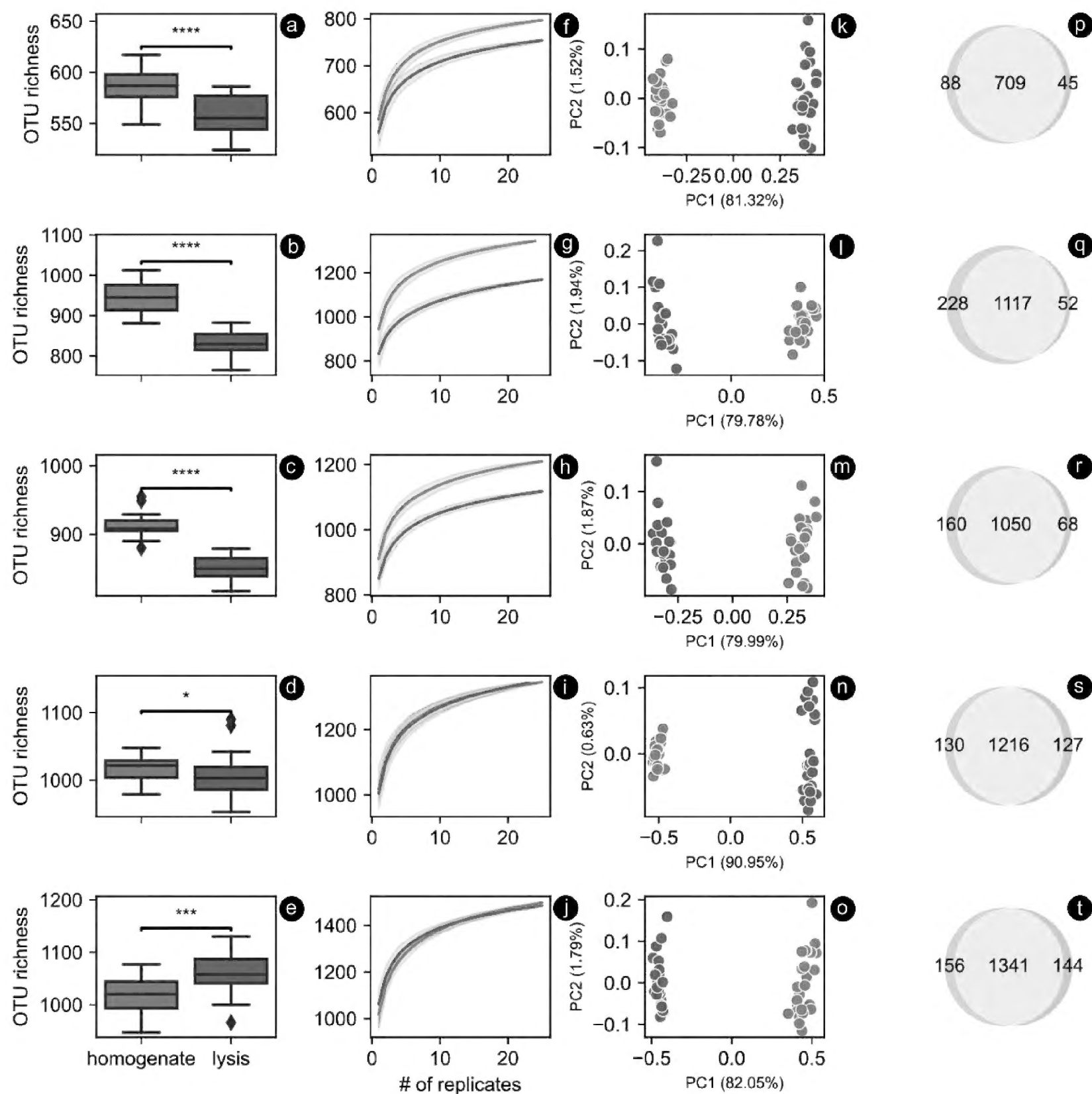
Figure 2. Comparison of different metrics for Malaise traps analyzed based on homogenized bulk samples (red) and via non-destructive lysis (blue). **a–e** OTU richness per sample, asterisks indicate significant differences between methods (Mann-Whitney-U test, p-value < 0.05 = *, p-value < 0.01 = **, p-value < 0.001 = ***, p-value < 0.0001 = ****). **f–j** OTU accumulation curve based on 25 technical replicates per sample and method. Curves show median values of all possible or max. 1000 random replicate draws for homogenate (red) and non-destructive lysis samples (blue). **k–o** PCA plots indicating community dissimilarity (Jaccard distance) of the 25 replicates per method. **p–t** Venn diagrams indicating OTU overlap as well as OTUs exclusively detected by each method.

samples (Fig. 2p–t). Still, the majority of OTUs were detected in both treatments, i.e. 80–84% of shared OTUs across all samples (Fig. 2p–t).

A comparison of the community similarity across all samples collected in 5 consecutive weeks in the same area reveals that both methods detect similar patterns in beta diversity irrespective of the treatment (Fig. 3).

Discussion

Our study revealed that in most cases metabarcoding of insect bulk samples from non-destructive lysis detected a smaller fraction of the community compared to homogenized samples of the same communities. This was true for the
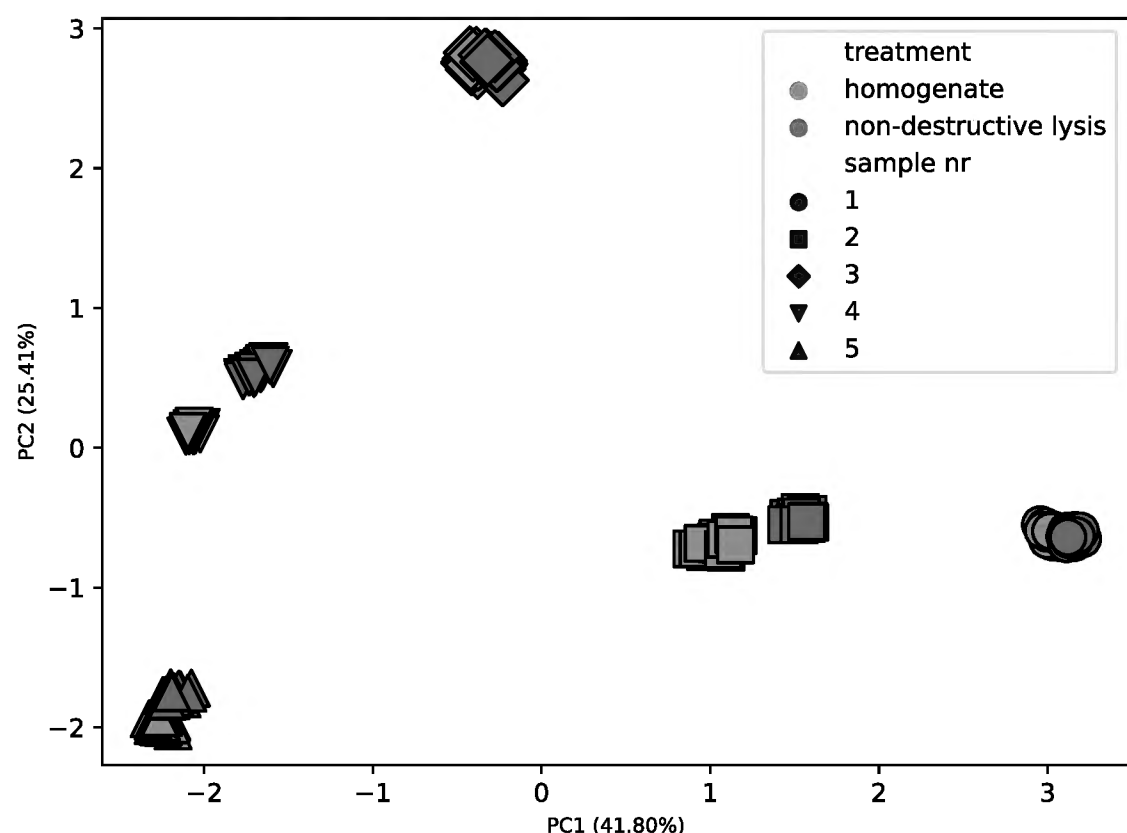
Figure 3. PCA plot indicating community dissimilarity (Jaccard distance) based on all five Malaise trap samples (different shapes), the two different methods (red = homogenate, blue = non-destructive lysis) and the 25 individual replicates per sample and method.

recovered OTU richness as well as for the number of OTUs exclusively detected by the two methods. We consider several possible reasons for the better performance of homogenization compared to non-destructive lysis:

Homogenization accesses DNA from strongly sclerotized or small specimens that may not release significant amounts of DNA into the lysis buffer during non-destructive lysis (Zizka et al. 2019; Martins et al. 2021; Martoni 2022). In this study, we could not detect patterns in OTU exclusiveness that would indicate taxonomic reasons (i.e. sclerotization) for detection likelihood (Suppl. material 5). Instead, exclusive OTUs detected primarily belonged to families with small body sizes in both treatments, indicating that probability of detection is lower for specimens with low biomass. In addition, the number of cells in contact with the lysis buffer is higher in homogenate compared to non-destructive lysis, which logically leads to an increased DNA yield when treated with identical protocols. This probably leads to reduced OTU recovery with non-destructive lysis due to fewer DNA molecules obtained compared to those extracted from homogenized tissue.

Furthermore, a lower concentration of DNA molecules resulting from non-destructive lysis may also lead to a dilution effect that decreases the probability of detection, especially for OTUs close to the detection limit of the method. Even though previous studies did not confirm an effect of lysate volume on OTU recovery (Nielsen et al. 2019), we think that this effect is likely to occur in non-destructive lysis as applied in this study. Since Nielsen et al. used low specimen numbers in low sample volumes with a very sensitive, high-yield Phenol-Chloroform DNA extraction, we think that direct comparability to our study is limited and that a dilution effect can be assumed in our case.

In addition, we assume that in non-destructive lysis, a certain amount of DNA is leached from the sample that would otherwise be available in the homogenate. Therefore, we expect that homogenization alone recovers an even more extensive community than when performed after non-destructive lysis as done in this study. However, this expectation cannot be validated with this study design, but must be considered, especially in experimental setups where DNA

degradation is a concern. Furthermore, in non-destructive lysis a certain number of small specimens fully disintegrated, leaving their recovery solely possible in non-destructive lysis but not in homogenization.

Although non-destructive lysis avoids homogenization, the morphological integrity of samples significantly deteriorated after 18 hours of incubation. For this study, a long incubation time was chosen for maximum DNA yield and consequently the highest possible recovery of OTUs (Marquina et al. 2022). In applications aimed at preserving morphological integrity, shorter incubation times may be advisable even though DNA yield is lower. This, however, may decrease the number of detected species with metabarcoding and thus a trade-off remains. An alternative could be to replicate sampling or split samples and use one half with a protocol optimized for preservation for morphological inspection and use the other half with a protocol optimized for DNA extraction. But this, in turn, leads to the effect that all species with only a single individual cannot be found in both fractions, reducing the recovered richness.

The direct comparison of both methods reveals similar ecological patterns, which is encouraging as it allows for comparability. However, non-destructive lysis has several important downsides to consider: The higher variability between communities recovered with non-destructive lysis as well as the relatively lower richness recovered indicate that study designs relying on non-destructive lysis should consider a higher number of replicates or the extraction of a larger lysate volume to achieve a comparable species recovery. Ideally, all of the lysate would be extracted to maximize species recovery. This, however, makes automated sample analysis very complicated or even impossible. Additionally, the high amounts of Proteinase K necessary for non-destructive lysis make this protocol rather expensive with a cost of approximately 40–60 € per sample depending on the sample biomass solely for the lysis of samples. This is already as expensive as the entire sample analysis in our regular workflow (Buchner et al. 2024). Lower concentrations of Proteinase K have also been applied in other studies (Porco et al. 2010), but this is expected to reduce the effectiveness of the non-destructive lysis and deviates from recommended working concentrations for commercially available kits. Furthermore, non-destructive lysis requires more manual labor and much longer handling times compared to homogenization. Therefore, we conclude that non-destructive lysis is not suitable for integration into (automated) standardized monitoring protocols soon. We are aware that previous studies have stated otherwise (Kirse et al. 2022), but think that methodological shortcomings like unequal sample volumes and different extraction methods per treatment limit the comparability of this study. Moreover, the benefits of non-destructive lysis for biodiversity monitoring and integrative taxonomy are not obvious: DNA metabarcoding is most useful in cases where morphological identification is impractical, i.e., for analysis of large numbers of hard to identify specimens. But due to the inability to link the species / OTU list generated via metabarcoding to individual specimens, this key problem persists after non-destructive lysis. Even if species / OTUs of interest are detected in the final lists, it will be very difficult and time-consuming to screen the samples for them, and often the morphology may also suffer from the treatment. However, in specific applications, e.g. museomics where preserving specimens' morphological integrity is crucial and the budget is sufficient, non-destructive lysis can still be of high value (Call et al. 2021; Salazar and Nattier 2020). In conclusion, homogenization offers a more comprehensive and cost-effective assessment of

Malaise trap insect communities compared to non-destructive lysis. Despite the higher costs, non-destructive lysis recovers at least 80% of the species found with homogenization, indicating that both methods can be intercalibrated and used in standardized biodiversity assessments depending on the specific objectives of a study. While non-destructive lysis has several limitations, including higher costs, greater manual labor, and longer handling times, making it less suitable for integration into automated standardized monitoring protocols, it can be invaluable for applications where preserving the morphological integrity of samples is crucial.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

LW, DB, and FL drafted the study design. JE conducted the sampling in the field as well as the sample sorting. DB and LW conducted the laboratory processing of the sampling and the bioinformatic analysis of the data. LW wrote the first draft of the manuscript, which was edited by all coauthors.

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Data availability

Demultiplexed raw read data for this publication are available via the European Nucleotide Archive (ENA) under the accession number PRJEB76617 (https://www.ebi.ac.uk/ena/browser/view/PRJEB76617).

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Supplementary material 1

Script S1

Authors: Lisa Wolany, Julian Enss, Florian Leese, Dominik Buchner
Data type: py
Explanation note: Code to reproduce the data analysis.

Link: https://doi.org/10.3897/mbmg.8.129814.suppl1

Supplementary material 2

Lysis buffer and subsequent ethanol volumes for the individual samples

Authors: Lisa Wolany, Julian Enss, Florian Leese, Dominik Buchner
Data type: xlsx

Link: https://doi.org/10.3897/mbmg.8.129814.suppl2

Supplementary material 3

Primers used for the first and second PCR in this study

Authors: Lisa Wolany, Julian Enss, Florian Leese, Dominik Buchner
Data type: xlsx
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Link: https://doi.org/10.3897/mbmg.8.129814.suppl3

Supplementary material 4

Equal sequencing depth for all replicates

Authors: Lisa Wolany, Julian Enss, Florian Leese, Dominik Buchner
Data type: xlsx
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Link: https://doi.org/10.3897/mbmg.8.129814.suppl4

Supplementary material 5

OTU exclusiveness

Authors: Lisa Wolany, Julian Enss, Florian Leese, Dominik Buchner
Data type: xlsx
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Link: https://doi.org/10.3897/mbmg.8.129814.suppl5